EXAMINATION OF GROWTH-INHIBITING SUBSTANCES SEPARATED BY PAPER CHROMATOGRAPHY IN FLESHY FRUITS
II. IDENTIFICATION OF THE SUBSTANCES OF GROWTH-INHIBITING ZONES ON THE CHROMATOGRAMS

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Introduction

In a previous paper (43) the ether-extractable growth-inhibiting substances of 26 various fleshy fruits were chromatographed and bio-assayed in order to establish their position on the chromatograms and from this to draw conclusions concerning their character. According to the results of these examinations 3 or 4 distinctly detectable inhibiting zones appeared on the chromatograms of all the fruits examined. In the sector below R_f 0,3 of the chromatograms of almost all the fruits one or two, but generally two, inhibiting zones exerting a milder activity could be observed which in many cases were replaced by mildly promoting spots. This area of the chromatograms was characterized by a great variety of inhibiting and promoting effects thus it seems likely that several substances are situated here the biological activity and properties of which differ.

In the range of R_f 0,60—0,85 (β-inhibitor zone) a strong inhibition appeared on the chromatogram of all the fruits. This suggests that either one very effective, or several very active inhibitors having a weak acidic character (summarized under the name of β-inhibitor complex) are closely located on this area of the chromatograms.

At the front line of the chromatograms (R_f 0,9—1,0) a very effective inhibiting zone presumably of neutral character was localized. It may be that also in this case several related substances are involved.

Concerning the nature of the substances of these active spots so far only assumptions have been made. In the course of the successive examinations reported in the present paper, however, the identification of the substances located in the inhibiting zones on the chromatograms of fruit juices will be attempted, as far as this can be realized in the frame of paper chromatography.

Materials and methods

The identification of the ether-extractable, biologically active substances of fruit juices was attempted in three fleshy fruits: *Citrus medica* L., *Prunus armeniaca* L. »Rakovszky«, and *Fragaria ananassa* Duch. »Eszterházy«. The examinations were begun in December 1956 with lemons and continued in the spring and summer of 1957 with strawberries and apricots.

3 kgm of the meso- and endocarpium of lemons, 5 kgm of the mesocarpium of apricots and 5 kgm of the fleshy tissues of strawberries were pressed out, extracted with ether and 20 cm ascending chromatograms were made from the condensed extracts with isopropanol: ammonia (sp. gr. 0,88): water 10 : 1 : 1 solvent, in the dark at 23° C, on Sch & Sch No. 2043 B filter paper, in the manner reported in a previous paper (43). 80 chromatograms of the ether extract of lemon juice and 120 of that of apricots and strawberries, with 3 start points each, were made. A part of these chromatograms were reserved for analysis in UV light and for spraying with different reagents, from the rest the strips containing the inhibiting spots were cut out and their substances eluated with methanol or ethanol. The eluata of the single spots were evaporated dry on a water bath of 50° C at reduced pressure and then dissolved with bidistilled water of known volume. With these standard solutions containing the concentrated substances of the active spots various chemical reactions and bioassays were performed, and some were rechromatographed in numerous other solvent systems.

In addition to the numerous chromatograms obtained from the ether extracts of lemons, apricots and strawberries, at least 1—2 chromatograms made in the course of the previous experiments (43) from other fruits were also available for the identification.

The estimation of the inhibiting spots was carried out on the basis of the relative and absolute R_f values observed in various solvent systems, the UV fluorescence, and the qualitative reactions given by spraying reagents, furthermore by comparison with the properties of the control synthetic compounds.

For development of the chromatograms the following solvent systems were applied (5, 7, 34).

Chromatographic systems involving alkaline and neutral solvents: ethanol: ammonia : water 90:5:5 and 80:5:15; n-butanol : ammonia : water 80:10:10 and 60:30:10; n-butanol saturated with water; and isopropanol : water 2:3.

Chromatographic systems involving acidic solvents: chloroform: 95% ethanol 1 : 1 + 2% (v/v) 90% formic acid; benzene: acetic acid: water 2 : 1 : 1; n-butanol: acetic acid : water 4:1:5; and 10% acetic acid.

Spraying reagents for detection and identification of the spots:

1. Bromcresol green (CRAMER 10)
2. Chlorophenol red (BROWN 7)
3. Ammonical silver nitrate (BUCH et al. c. f. 33)
4. 10% (v/v) acetic anhydrine in pyridine (BUCH et al. c. f. 33)
5. 0,5% methanolic ferric chloride (SCHMIDT 35)
6. Diazotized sulphanilic acid (MANN c. f. 28)
7. Diazotized p-nitroaniline (BRAY c. f. 5)
8. Diazotized benzidine (KOCH and KRIEG c. f. 39)

For biological tests like in the previous papers wheat coleoptile sections (*Triticum vulgare* L. »Bánkúti 1201«) and poppy seeds (*Papaver somniferum* L. »Fertődi kék«) were used.

Experimental results

A) Chemical estimation of the substances of the active spots observed between Rf 0,0—0,3.

To establish the position of the short chain organic acids (mainly di- and tricarboxylic acids) occurring in relatively large amounts in fruit juices many such compounds were chromatographed under identical conditions as the fruit juices (isopropanol : NH_3 : water 10:1:1). The results suggest that the first of the lower inhibiting spots (R_f 0,0—0,1) observed on the bio-assayed fruit chromatograms is produced by malic-, tartaric- and citric acid while the second (R_f 0,15—0,20) mainly by succinic- and ascorbic acid. This assumption is supported by the fact that on all these areas of the chromatograms sprayed with pH indicator bright yellow spots appeared indicating the presence of strong acids.

For the identification of the substances of these acidiferous spots their methanolic eluate was rechromatographed in numerous other solvent systems. The R_f values, the fluorescence and the colour reactions with ammoniacal silver nitrate and with acetic anhydride in pyridine, furthermore, the results obtained by simultaneous running of pure acids showed beyond doubt that the two lower inhibiting spots of the chromatograms of lemons and apricots contain citric-, malic-, tartaric-, succinic and ascorbic acid as well as other related acids. For elucidation of the biological activity of these acids a range of concentration of 10^{-1} M to 8.10^{-5} M was prepared and bio-assayed. According to the results all the acids examined exert in higher concentrations (up to about 10^{-3} M) an inhibitory effect whereas in lower concentrations (up to about 2.10^{-4} M) they promote the growth of coleoptile sections and Papaver embryos, however, in similar concentrations the activity of the single acids varies too. Thus it is obvious that in these positions and on the adjacent areas of the chromatograms of the fruits — depending on the concentration — both inhibiting and promoting zones occurred (43).

The tannic acids are also located in the R_f 0,0—0,3 sector of the chromatograms. These substances were estimated by their characteristic chemical reactions and by comparison with known compounds. The tannic acids inhibit growth and germination only in higher concentrations, in lower concentrations on the other hand, their effect turns into promotion. The activity they exert is less considerable than of the short chain carboxylic acids.

The position and biological activity of caffeic- and chlorogenic acid was dealt with in detail. For the identification of these substances not only the R_f values and the fluorescence were considered but spraying reactions with ferric chloride, KOH, HÖPFNER's reagent (37) and ethanolic phosphomolybdic acid (5) were also applied. On the fruit chromatograms caffeic acid (R_f 0,17) could only be detected rarely and in very small amounts whereas chlorogenic acid (R_f 0,25) appeared frequently. For comparison these compounds were isolated according to the method of STEVENS and NORD (37) from coffee beans. The biological examinations of various concentrations of these two acids indicated that their activity is fairly low.

B) The identification of the substances of the β-inhibitor zone.

In order to get acquainted with the nature of the substances located in the β-inhibitor zone (R_f 0,55—0,85) first of all their physico-chemical properties were examined. The results show that the substances of the β-inhibitor spot are soluble in water, ether, methanol, ethanol and acetone, and practically insoluble in chloroform, petrolether and benzene, they are thermostable as well

as sensitive to alkalines and peroxides. The spraying with pH indicators proved the weak acidic character of these compounds, however, a comparison of the pH and the biological activity of the eluate rendered evident that the inhibiting activity of the substances participating in the creation of the β-inhibitor zone can by no means be attributed to the acid effect.

Even if the eluate of the β-inhibitor zone is highly diluted it does not promote the elongation of the coleoptile sections and the *Papaver* roots, thus these compounds do not contribute to the stimulating effect exerted by highly diluted fruit juices (12, 44). Hence, it may be assumed that the growth-promoting activity of diluted fruit juices is due to the fact that the inhibiting action of the components of the β-inhibitor complex ceases, whereas the promotion characterising the low concentrations of aliphatic acids and tannic acids comes into prominence.

As regards the chemical determination of the β-inhibitor spot, because of its weak acidic character and its position on the chromatogram, it could be anticipated that it is a mixture of several aromatic acids with similar R_f values. To gather inform what kind of chemicals are involved all the aromatic acids available were chromatographed under similar conditions as the fruit extracts to establish and compare their R_f values. As numerous aromatic acids, mainly which contain a phenolic group, run onto the area corresponding to the β-inhibitor zone it was assumed that the substances of the β-inhibitor complex of fruits are also benzene or phenol derivatives.

For separation of the substances located in the β-inhibitor zone the eluate of the spot was rechromatographed in many other solvent systems. Analyses in UV light, spraying with various reagents and biological examinations revealed that the β-inhibitor zones of lemons and strawberries contain three inhibitors (C_1, C_2, C_3 and F_1, F_2, F_3 respectively), and that of apricots four inhibitors (P_1, P_2, P_3, P_4). For chemical determination of these substances, besides spraying with different phenol reagents, the GORDON—WEBER's indole test and the FEIGL's lactone test was also applied. The results of these experiments are summarized in Table I.

According to the data of Table I the component of the β-inhibitor of lemons and strawberries showing a violet fluorescence is identical with salicylic acid and its ammonium salt formed during the chromatography, respectively. This is also proved by the similarity of the behaviour of crystalline salicylic acid added to the eluate of the spots.

The properties of the C_2, F_2 és P_2 inhibitors show unequivocally that they are o-coumaric acids. It seems probable that o-coumaric acid forms in alkaline solvents partly or perhaps wholly from coumarin which has already been found by several authors in fruit juices and the strong inhibiting activity of which is well known. Namely, the lactone ring of coumarin which is a δ-lactone, opens readily in alkaline solutions to give the corresponding hydroxy-acid (16). This assumption is supported by the fact that in the case of apricots the eluate of this spots gave a positive lactone test. Namely, on the apricot chromatograms the spot of P_2 inhibitor was the largest, it also showed the brightest fluorescence and gave the most intensive colour reactions, its concentration being the highest on the chromatograms. This explains why the eluate of the P_2 inhibitor was the ordy one which indicated a discernible lactone reaction demon-

Table I. Identification of the substances of the inhibiting spots separated from the β-inhibitor complex by ethanol: ammonia: water (80 : 5 : 15) solvent.
(+ = positive reaction; − = negative reaction; blank = no treatment)

No	Inhibitor	UV fluorescence		Spraying reagents							R_f values				Identification
		untreated	+ NH_3 vapour	bromcresol-green	$FeCl_3$ in $HClO_4$	Feigl-test	0,5% methanolic $FeCl_3$	diazotized sulphanilic acid	diazotized p-nitroaniline + Na_2CO_3	diazotized benzidine + Na_2CO_3	1	2	3	4	
											solvent system*				
1.	C_1 inhibitor	violet	violet	+	−	−	violet	−	yellow	yellow	0,82	0,95	0,84	0,64	salicylic acid
2.	C_2 inhibitor	bluish green	bright greenish yellow	+	−	−	yellow		faint violet	faint red	0,84	0,90	0,73	0,58	o-coumaric acid
3.	C_3 inhibitor	faint blue	bright blue	+	−	−	faint yellowish brown		faint greyish blue	violet	0,89	0,86	0,57	0,49	p-coumaric acid or ferulic acid
4.	F_1 inhibitor	violet	violet	+	−	−	violet	−	faint yellow	yellow	0,81	0,95	0,85	0,63	salicylic acid
5.	F_2 inhibitor	bluish green	bright greenish yellow	+	−	−	yellow		violet	reddish	0,85	0,91	0,73	0,39	o-coumaric acid
6.	F_3 inhibitor	blue	blue	+	−	−	−	−	faint yellow	faint brown	0,90	0,92	0,93	0,59	cinnamic acid?
7.	P_1 inhibitor	violet	violet	+	−	−	−	red			0,78	0,94	0,78	0,63	?
8.	P_2 inhibitor	yellowish green	very bright greenish yellow	+	−	+	yellow	−	violet	reddish	0,84	0,91	0,72	0,57	o-coumaric acid and coumarin
9.	P_3 inhibitor	blue	blue	+	−	−	faint brownish violet	−	red		0,76	0,91			m-oxy-benzoic acid?
10.	P_4 inhibitor	−	−	+	−	−	−	yellowish	−	−	0,74	0,85			?

* Solvent systems: 1. ethanol: ammonia: water 80 : 5 : 15; 2. n-butanol: acetic acid: water 4 : 1 : 5; 3. n-butanol; water; 4. 10% acetic acid

strating the presence of coumarin. Moreover, the presence of coumarin in this spot was also recognizable by its characteristic odour. On the other hand, the colouration and fluorescence of the spot already pointed to o-coumaric acid suggesting the presence of both o-coumaric acid and coumarin. It may be that the corresponding spots of lemons and strawberries also contain coumarin, however, owing to its low concentration it could not reach the limit of susceptibility of FEIGL's test.

The colour reactions and the position of C_3 inhibitor suggest that it is either p-coumaric acid or ferulic acid, i. e. the behaviour of these two cinnamic acid derivatives is very similar. It may, however, also be that this inhibiting spot is formed by the two compounds together, though the attempt to separate them was not successful.

The exact quality of the F_3 inhibitor could not be established. Owing to its position and chemical reactions numerous aromatic acids may be taken into consideration, however, because of its colouration with p-nitroaniline cinnamic acid seems the most probable.

The P_1 inhibitor shows an entirely identical fluorescence to that of the spot established as salicylic acid and its R^f value also approaches that of the latter, but in the course of its treatment with spraying reagents quite different qualities could be observed. As owing to technical reasons further examinations could not be carried out it can only be established that P_1 inhibitor is also a phenolic compound.

In spite of numerous attempts the P_4 inhibitor could not be determined as the procedures with various spraying reagents did not furnish any positive data.

The results of the above examinations were also confirmed by the comparison of the behaviour and biological activity of the corresponding synthetic compounds[1].

C) Identification of the substances of the inhibiting zone along the front line.

In the course of the bio-assay of the various fruit chromatograms just under the front line (R_f 0,9—1,0) a strong inhibition could be detected (43). The question arises which substances are responsible for this inhibiting activity.

During the examinations of the freshly developed chromatograms a yellowish, transparent oil strip, emitting the characteristic odour of the fruit, was striking in every case along the front line. These observations suggest that this very effective inhibiting zone contains the neutral odoriferous substances of the fruit, the so-called essential oils. The presence of these substances was also confirmed by the fact that after airing the chromatograms for a few days an inhibition exceeding the standard error could no more be observed in this area, and that on the chromatograms obtained from boiled extracts this inhibiting zone has disappeared (43).

To provide further evidence germination tests were carried out with *Sinapis* and *Papaver* seeds in vapour of paper strips cut out of the front line of the chromatograms (44, 45). The seeds exposed to the vapours of these paper strips

[1] Ferulic acid and p-coumaric acid were isolated from the bark of the trunk of *Catalpa bignonioides* Walt. according to the method discribed by STEVENS and NORD (37).

exhibited a strong germination and growth inhibition (Table II) which is a characteristic property of the volatile oils (1, 12, 18, 41, 44).

Table 2.

Percentage of germination and root-growth of *Sinapis* and *Papaver* seeds in the presence of essential oils of strawberries, apricots and lemons. (The average of four parallel examinations.

Chromatogram strips R_f 0,9---1,0	Percentage of germination				Percentage of root-growth of the seedlings 60 hours	
	after 24 hours incubation		after 60 hours incubation			
	Sinapis	Papaver	Sinapis	Papaver	Sinapis	Papaver
Control	100	100	100	100	100	100
Strawberries	3	2	41	28	18	22
Apricots	5	4	56	57	37	23
Lemons	4	3	60	61	36	41

Discussion

A) The role of the short chain carboxylic acids and tannic acids in the inhibiting activity of fruit juices.

The greater part of the investigators dealing with the role of short chain organic acids in the inhibiting effect of fruit juices (12, 21, 22, 23, 24) state that these acids, although they actually exert an inhibiting effect, can after all not be the principal inhibiting agents of fruit juices. Our paperchromatographic examinations support this view as in the course of the bio-assays it became evident that the effectiveness of these acids is far less marked than that of the members of the β inhibitor complex and the essential oils.

The results show that in higher concentrations citric-, malic-, tartaric-, succinic- and ascorbic acid have an inhibiting effect and that in low concentrations, on the contrary, they promote the growth of the coleoptile sections and *Papaver* embryos. This is in good agreement with the observation that in some less acidic fruit juices these compounds — obviously owing to their lower concentration — do not inhibit but rather stimulate elongation. The fact that several acids exert such a double activity has already been reported (4, 8, 15). The stimulating effect of short chain carboxylic acids in low concentrations may be due to the role they play in the *Krebs* cycle.

The inhibiting activity of tannic acids is also known (20, 25, 32, 36, 40). Our data concerning the effect of tannic acids are partly in accordance with those of the above authors, as far as in higher concentrations these acids actually inhibit growth and germination. According the results of the biological examinations performed with numerous fruits, however, it can be stated that no significant inhibiting effect may be attributed to the amount of tannic acids generally contained in fruit juices, moreover, owing to their fairly low concentration they often even promote growth.

The role played by caffeic- and chlorogenic acids was dealt with separately. It is known that AKKERMANN and VELDSTRA (1) postulate that caffeic acid is partly responsible for the strong inhibiting activity exerted by the acidic fraction of ether extracts of tomatoes. MAYER and EVENARI (31) on the other hand, are of the opinion that the effectiveness of this acid is too weak to play an important role in the fruit juice. Recently on analysing the juice of 10 fleshy fruits HERMANN (17) concluded hat they do not contain, or only in insignificant amounts caffeic acid.

Our results concerning caffeic acid are in agreement with those of the two latter workers. We could only detect this substance on a few chromatograms and on the areas where caffeic acid was located the bio-assays never showed an appreciable inhibition. Chlorogenic acid occur in fleshy fruits far more frequently than caffeic acid, this fact has also been reported by HERMANN (17). Chlorogenic acid, however, in spite of its wide spread occurence, does not play an important part in the inhibiting activity of fruit juices as in the course of the bio-assays it became obviously that the action due to this compound is rather negligible.

Possibly in addition to aliphatic acids and tannic acids other substances also contribute to a certain extent to the inhibiting effect appearing on the lower sector of the fruit chromatograms. Whatever kind of substances may be involved they cannot be important growth inhibitors.

B) Discussion of the results obtained concerning the β-inhibitor complex.

KÖCKEMANN (23) described in 1934 a water-, ether-, ethanol- and acetone soluble, in benzene insoluble, non volatile, thermostable inhibitor of acidic character, considered by him to be the main inhibitory factor of the fruit juices. He termed this unknown inhibitory agent blastocholine. Later it was revealed that blastocholine is not a single compound (27) but for a few exceptions (1, 26) chemical estimation has not been carried out.

At the investigation of the physico-chemical properties of the substances included in the β-inhibitor zone it became evident that they agree completely with those KÖCKEMANN reported regarding the blastocholine. Consquently it seems beyond doubt that the socalled β-inhibitor complex of fruit juices and the inhibitory factor termed blastocholine by KÖCKEMANN are identical.

Further examinations proved that the considerable width and the shift of the β-inhibitor zone is not caused by tail formation and/or fluctuation of the R_f values due to experimental errors, but in the first place by the circumstance that the β-inhibitor zone contains several related compounds. It could also be established that the composition of this complex and the quantitative proportion of its components are not identical in the various fruit species. But in all cases it became clear that the inhibitors separated from this zone are related compounds belonging to the benzoic- and cinnamic acid derivatives and thus in a wider sense, the β-inhibitor complex of fruits may be considered to be uniform.

The growth-inhibiting activity and natural occurence of the substances identified in the β-inhibitor zone of fruits is already known. Among these compounds coumarin and salicylic acid are the most effective. As compared with them, the other components (ferulic acid, coumaric acids and m-oxybenzoic

acid) do not exert one by one a considerable inhibition, howewer, their interaction — owing to the well known synergistic effect — may after all be significant (31). At the bio-assays of the fruit chromatograms the resultant of the action of these substances could be observed (43).

STOWE et al (38) assumed that the β-inhibitor is an indolic or phenolic acid. The results presented here justify the latter assumption concerning fleshy fruits, but contradict the participation of indole compounds, inasmuch as the condensed eluate of the β-inhibitor spots of the fruit chromatograms did not in a single case give a positive indol reaction (Table I).

Recently various benzoic- and cinnamic acid derivatives have been indentified in acid fraction of ether extract of other plant organs too. BÖRNER (6) determined p-oxy-benzoic acid, p-oxy-cinnamic acid and ferulic acid from the straw of cereals, MASSART (30) p-oxy-benzoic acid, ferulic acid, p-coumaric acid and vanillic acid from seed-balls of beets, and GRIFFITHS (14) ferulic acid, o- and p-coumaric acid as well as salicylic acid from *Theobroma cacao L.* According to the paperchromatographic examinations of KÖVES (25) the growth- and germination inhibitors of oat husks proved to be also cinnamic- and benzoic acid derivatives. These results suggest that the substances of the β-inhibitor complex determined in fruit juices in our experiments and the compounds of the β-inhibitor zones in the same position observed on chromatograms of plant organs other than fruits (2, 3, 19, 42) are related, i. e. the latters may also be cinnamic- and benzoic acid derivatives.

It seems very probable that in addition to the substances determined or suggested in the present paper, other compounds are also involved in the inhibiting complex of fruit juices examined. The complete separation and identification of all the components, however, cannot be accomplished in the scope of paper chromatography. This task requires far more material and other methods. The detailed analysis of the β-inhibitor complex of some fruits is in progress in our institute.

C) The role of essential oils in the activity of fruit juices.

The bacteriostatic and growth-inhibiting acitvity of volatile oils is commonly known since a long time (12, 36, 41) and several authors (1, 11, 18, 22, 44, 45) have also pointed out the inhibiting role they place in fruit juices. The results of our paperchromatographic examinations are in good accordance with the above establishments. In view of the findings it may be stated that the effectiveness of these substances in fresh fruit tissues is about to equal to that exerted by a mixture of benzoic- and cinnamic acid derivatives. The chemical composition of essential oils is extremely heterogenous. The main inhibiting role of their various components can particularly be attributed to the aromatic ketones and aldehydes, and to the free or etherified phenols and phenylpropane derivatives (12, 36).

Summary

It was attempted to identify the substances of the inhibiting spots of paper chromatograms made with the ether extract of fruit juice of lemons, strawberries and apricots. The detection and estimation of the inhibitors were performed on the basis of the UV fluorescence, the R^f values observed in numerous

solvent systems, the colour reactions obtained with different spraying reagents and finally by comparison with the properties of the corresponding synthetic chemicals.

In the active spots observed on the chromatograms between R_f 0,0—0,3 short chain carboxylic acids and tannic acids were found. According to their concentration these compounds inhibit or promote the growth of coleoptile sections and the germination of *Papaver* seeds.

It seems that aliphatic acids and tannic acids do not play an important role in the inhibition of fruit juices, on the contrary, owing to their fairly low concentration they rather promote than inhibit growth and germination. Because of their relative slight effectiveness caffeic- and chlorogenic acid can in no case be considered to be one of the main inhibitory factors in fruit juices.

The large inhibiting area observed on the chromatograms between R^f 0,55—0,85 (termed β-inhibitor zone) consists of several aromatic acids and their derivatives, respectively, located closely in these spot (β-inhibitor complex). According to its physico-chemical properties and biological activity the β-inhibitor complex can be identified with KÖCKEMANN's blastocholine. The composition and quantitative proportion of the components of this complex are not identical in the various fruit species.

In the β-inhibitor zone of the three fruits examined coumarin, o- and p-coumaric acid, ferulic acid and presumably cinnamic acid could be identified as cinnamic acid derivatives, whereas salicylic acid and possibly m-oxy-benzoic acid as benzoic acid derivatives. From the β-inhibitor complex of apricots in addition still two unknown aromatic acids could be separated. The action of these substances is synergistic.

The inhibiting zone along the front line (R 0,9—1,0) contains the essential oils of the fruits. The inhibiting effect of essential oils is about equal to that of the β-inhibitor complex, i. e. it represents one of the main inhibiting factors in the fruit juices.

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